REMARKS

Applicants respectfully request entry of these amendments. The amendments present the rejected claims in better form for consideration on appeal or comply with the requirements of form and, therefore, may be entered pursuant to 37 CFR §1.116. After entry of the amendment, claims 1 and 3-6 are pending. Claim 2 has been cancelled without prejudice or disclaimer. Amendments to the claims find support *inter alia* in the original claims. No new matter has been added.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 1 and 3-6 are rejected under 35 U.S.C. § 112, second paragraph, for allegedly being indefinite. The Examiner alleges that the set of purified peptides identified in Table 1 or 2 of the specification are not physical peptides and that the Applicants have not made compositions comprising the peptides of Table 1 or 2. Applicants respectfully disagree.

Tables 1 and 2 show the MS atomic mass unit identities of elastin peptides resulting from MMP12 digestion and separation by column chromatography. Page 45, lines 23-26. The set of purified peptides was prepared by degrading elastin with the enzyme MMP12 and subjecting the samples to a reversed phase preparation step before MALDI-TOF mass spec analysis. Page 40, lines 6-19. Therefore the peptide masses listed in Tables 1 and 2 correspond to the peptides produced by MMP12 digestion and reversed phase preparation.

Furthermore, the claims distinctly and definitely identify physical peptides by reciting both that the peptides are the degradation products of elastin and that they have a peptide mass identified in Table 1 or 2. There is no requirement that a peptide be identified by amino acid sequence. However, the amino acid sequence also is readily obtainable from the data provided.

From the known amino acid sequence of elastin, the amino acid sequences of the peptides listed in Tables 1 and 2 can be determined from the mass data using publicly available mass spectra sequence analysis algorithms. For example, Appendix A shows the amino acid sequences of the peptides of Table 1 determined from the peptide mass (observed mass) listed in Table 1 and the corresponding calculated mass determined by mass spectra sequence algorithms.

The difference between the observed mass and calculated mass is less than 0.25 mass units for each peptide. Thus a person of ordinary skill in the art would recognize the subject matter claimed with the particularity and distinctness required by 35 U.S.C. § 112.

The Examiner further alleges that some of the molecular weights listed in Table 1 or 2 correspond to molecules that are fragments of the MMP12 digestion products produced by the ionization process in mass spec. Applicants respectfully disagree. The type of mass spectrometry used in the present invention, MALDI-TOF, does not create peptides, but rather ionizes existing peptides to allow their atomic mass to be measured. Therefore MALDI-TOF is a preferred method of peptide analysis because peptides tend to fragment when ionized by other ionization techniques (see page 1 of MALDI-TOF entry in ChemWiki: The Complete Chemistry Textbook, at http://chemwiki.ucdavis.edu/Analytical_Chemistry/Instrumental_Analysis/MALDI-TOF, copy attached).

Because one skilled in the art would readily understand what is claimed, the claims are not indefinite. Reconsideration and withdrawal of the rejection is respectfully requested.

Rejections under 35 U.S.C. § 102

Claim 1 is rejected under 35 U.S.C. § 102(b) as being allegedly anticipated by Kucich et al. (WO91/18920; hereinafter "Kucich") as evidenced by Taddese. The Examiner alleges that Kucich discloses elastin fragments produced by digesting human elastin with human macrophage/neutrophil elastase (MMP12). Applicants strongly disagree. Kucich discloses human lung elastin digested with human neutrophil elastase. Page 6, Part II. Human neutrophil elastase is not equivalent to the matrix metalloproteinase 12 (MMP12) used in the present invention. As shown in the attached Appendix B, the amino acid sequence of human neutrophil elastase shares no significant sequence homology with the amino acid sequence of MMP12. Therefore, the Examiner has failed to point out where Kucich discloses a set of purified peptides comprising peptide products resulting from the degradation of elastin by the enzyme MMP12 as recited in the present claims.

Furthermore, the Patent Office relies upon Taddese as evidence that the elastin fragments of Kucich have the same peptide mass as those presently claimed. Yet Taddese cannot be used as evidence of the fragments prepared by Kucich. Because Kucich used elastin digested with human neutrophil elastase and Taddese reports elastin digested with MMP12, and the two are different, the resulting peptides need not be the same. Accordingly, Taddese fails to expand upon the peptides produced in Kucich and is not relevant.

Furthermore, the present application claims priority to Swedish Application No. 03025590 published September 25, 2003 whereas the Taddese reference was published in 2008. Therefore Taddese does not anticipate the present claims.

For these reasons, reconsideration and withdrawal of the rejection is respectfully requested.

CONCLUSION

In view of the present amendment and further in view of the above remarks, Applicants respectfully request withdrawal of the rejections and allowance of the claims. If any outstanding issues remain, the Examiner is invited to telephone the undersigned at the number given below.

Accompanying this response is a petition for a three-month extension of time, and Notice of Appeal, with the required fee payments. No further fee is believed due. However, if an additional fee is due, the Director is authorized to charge our Deposit Account No. 03-2775, under Order No. 15652-10100-US from which the undersigned is authorized to draw.

Dated: September 21, 2010

Respectfully submitted,

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Appendix A

Human elastin protein reference sequence registered on public repository as UniProtKB/Swiss-Prot P15502 (ELN_HUMAN)

aa position	sequence				
1	MAGLTAAAPR	PGVLLLLLSI	LHPSRPGGVP	GAIPGGVPGG	VFYPGAGLGA
51	LGGGALGPGG	KPLKPVPGGL	AGAGLGAGLG	AFPAVTFPGA	LVPGGVADAA
101	AAYKAAKAGA	GLGGVPGVGG	LGVSAGAVVP	QPGAGVKPGK	VPGVGLPGVY
151	PGGVLPGARF	PGVGVLPGVP	TGAGVKPKAP	GVGGAFAGIP	GVGPFGGPQP
201	GVPLGYPIKA	PKLPGGYGLP	YTTGKLPYGY	GPGGVAGAAG	KAGYPTGTGV
251	GPQAAAAAAA	KAAAKFGAGA	AGVLPGVGGA	GVPGVPGAIP	GIGGIAGVGT
301	PAAAAAAAAA	AKAAKYGAAA	GLVPGGPGFG	PGVVGVPGAG	VPGVGVPGAG
351	IPVVPGAGIP	GAAVPGVVSP	EAAAKAAAKA	AKYGARPGVG	VGGIPTYGVG
401	AGGFPGFGVG	VGGIPGVAGV	PSVGGVPGVG	GVPGVGISPE	AQAAAAAKAA
451	KYGAAGAGVL	GGLVPGPQAA	VPGVPGTGGV	PGVGTPAAAA	AKAAAKAAQF
501	GLVPGVGVAP	GVGVAPGVGV	APGVGLAPGV	GVAPGVGVAP	GVGVAPGIGP
551	GGVAAAAKSA	AKVAAKAQLR	AAAGLGAGIP	GLGVGVGVPG	LGVGAGVPGL
601	GVGAGVPGFG	AGADEGVRRS	LSPELREGDP	SSSQHLPSTP	SSPRVPGALA
651	AAKAAKYGAA	VPGVLGGLGA	LGGVGIPGGV	VGAGPAAAAA	AAKAAAKAAQ
701	FGLVGAAGLG	GLGVGGLGVP	GVGGLGGIPP	AAAAKAAKYG	AAGLGGVLGG
751	AGQFPLGGVA	ARPGFGLSPI	FPGGACLGKA	CGRKRK	

Appendix A (cont.)

peptide number	observed mass	calculated mass	Sequence and (adjacent aa)	position in P15502
1	772.4	772.3836	(V)GISPEAQA(A)	436-443
2	798.4	798.4104	(V)GPQAAAAAAA(K)	250-260
3	802.4	802.4166	(G)ADEGVRR(S)	611-619
4	817.5	817.4203	(L)GVGAGVPGFG(A)	601-610
5	823.4	823.3985	(K)LPYGYGPG(G)	225-233
6	824.4	824.3937	(P)GGVFYPGAG(L)	39-47
7	865.4	865.4050	(P)GGVADAAAAY(K)	91-100
8	874.5	874.5033	(P)AVTFPGALV(P)	84-92
9	878.5	878.5094	(A)KAAKYGAAV(P)	653-661
10	904.4	904.4887	(K)AAKYGAAVPG(V)	654-663
11	937.5	937.5102	(A)PGIGPGGVAAAA(K)	546-557
12	944.5	944.5200	(A)AQFGLVPGVG(V)	49-507
13	951.5	951.4934	(G)KLPYGYGPG(G)	225-233
14	977.5	977.5091	(G)AFPAVTFPGA(L)	81-90
15	1027.5	1027.5531	(I)GPGGVAAAAKSAA(K)	548-561
16	1072.6	1072.5898	(A) GQFPLGGVAAR(P)	751-760
17	1073.6	1073.5990	(G)LGAGLGAFPAVT(F)	78-86
18	1089.5	1089.5647	(G)ADEGVRRSLS(P)	611-622
19	1094.6	1094.5840	(G)VPGTGGVPGVGTP(A)	474-486
20	1107.6	1107.6157	(A)AGVLPGVGGAGVPG(V)	271-284
21	1114.6	1114.6255	(K)AAQFGLVPGVGV(A)	497-508
22	1137.6	1137.5899	(V)PGTGGVPGVGTPAA(A)	472-487
23	1141.6	1141.5848	(P)GVGISPEAQAAAA(A)	433-446
24	1142.6	1142.6800	(P)TGTGVGPQAAAAAA(A)	246-259
25	1145.6	1145.5990	(R)PGFGLSPIFPGG(A)	763-774
26	1155.6	1155.6004	(G)VGISPEAQAAAAA(K)	431-447
27	1171.6	1171.6000	G)LAGAGLGAGLGAFP(A)	69-83
28	1185.7	1185.6950	(G)GVAAAAKSAAKVAA(K)	552-565
29	1193.6	1193.6000	(L)PYGYGPGGVAGAAG(K)	227-240
30	1199.6	1199.5903	(R)SLSPELREGDP(S)	620-630
31	1216.6	1216.5705	(G)AGVPGFGAGAD(Acetohydrazide)E(Acetohydrazide)G(V)	604-616
32	1232.6	1232.627	(V)PGGVADAAAAYKAA(K)	93-106
33	1237.6	1237.6535	(G)GVPGVGISPE(Acetohydrazide)AQA(A)	431-443
34	1242.7	1242.6913	(V)RRSLSPELRE(G)	618-627
35	1254.7	1254.6841	(G)AGVPGLGVGAGVPGF(G)	595-609
36	1255.9	1255.7157	(G)AGVKPKAPGVGGAF(A)	173-186
37	1262.6	1262.6528	(I)PGVGPFGGPQPGVP(L)	190-203
38	1265.7	1265.6848	(L)GVSAGAVVPQPGAGV(K)	122-136
39	1285.7	1285.6899	(V)PGGLAGAGLGAGLGAF(P)	67-82
40	1287.7	1287.7056	(A)AQFGLVGAAGLGGLG(V)	699-713
41	1291.7	1291.7005	(A)GVPSVGGVPGVGGVPG(V)	419-434
42	1296.7	1296.6906	(I)GGIAGVGTPAAAAAAAA(A)	293-309
43	1298.7	1298.6964	(L)HPSRPGGVPGAIPG(G)	22-35
44	1299.7	1299.7056	(K)AAKYGAAAGLVPGGP(G)	313-327
45	1314.7	1314.7165	(I)PPAAAAKAAKYGAAG(L)	729-743
46	1320.8	1320.811	(S)PRVPGALAAAKAAK(Y)	643-656
47	1322.7	1322.7427	(G)VPGVGTPAAAAAKAAA(K)	480-495

Appendix A (cont.)

48	1331.7	1331.6954	(L)VPGGVADAAAAYKAA(K)	92-106
49	1347.7	1347.7056	(G)VGAGGFPGFGVGVGGI(P)	399-414
50	1352.7	1352.6593	(P)YGYGPGGVAGAAGKAG(Y)	228-243
51	1363.7	1363.7005	G)GVFYPGAGLGALGGGA(L)	40-55
52	1368.8	1368.7998	(G)VKPKAPGVGGAFAGI(P)	175-189
53	1369.0	1368.7998	(G)VKPKAPGVGGAFAGI(P)	175-189
54	1370.7	1370.7314	(P)AVTFPGALVPGGVAD(A)	84-98
55	1379.6	1379.7641	(L)GGLGVGGLGVPGVGGLGG(I)	710-727
56	1402.8	1402.8053	(G)AGVPGVPGAIPGIGGIA(G)	280-296
57	1424.8	1424.822	(P)GIGPGGVAAAAKSAAKV(A)	547-563
58	1440.7	1440.7958	(T)GAGVKPKAPGVGGAFAG(I)	172-189
59	1455.8	1455.8027	(E)GVRRSLSPELREG(D)	616-628
60	1469.8	1469.8111	(G)LGGVLGGAGQFPLGGVA(A)	744-760
61	1477.8	1477.8122	(I)PGGVVGAGPAAAAAAAKAA(A)	677-695
62	1484.8	1484.822	(I)PPAAAAKAAKYGAAGLG(G)	629-645
63	1505.8	1505.7958	(V)GGVPGVGGVPGVGISPEA(Q)	424-421
64	1508.9	1505.8798	(P)GVGGLGGIPPAAAAKAAK(Y)	721-738
65	1519.8	1519.8115	(P)GAGIPGAAVPGVVSPEAA(A)	356-373
66	1520.8	1520.8067	(P)QAAVPGVPGTGGVPGVGT(P)	468-485
67	1524.8	1524.8169	(L)GVGAGVPGLGVGAGVPGFG(A)	591-610
68	1536.8	1536.8016	(P)GVGGVPGVGISPEAQAAA(A)	428-445
69	1542.8	1542.8387	(V)GPQAAAAAAAKAAAKFGA(G)	251-268
70	1570.7	1570.8296	(E)GVRRSLSPELREGD(P)	616-629
71	1596.8	1596.8897	(G)LPGVYPGGVLPGARFP(G)	146-161
72	1599.8	1599.8602	(V)GPQAAAAAAAKAAAKFGAG(A)	251-269
73	1613.9	1613.901	(A)PGVGVAPGVGVAPGIGPGGV(A)	534-553
74	1644.8	1644.834	(P)GGVAGAAGKAGYPTGTGVGP(Q)	233-252
75	1666.9	1666.8911	(F)YPGAGLGALGGGALGPGGKP(L)	43-62
76	1670.9	1670.9013	(G)QFPLGGVAARPGFGLSP(I)	752-769
77	1687.9	1687.9014	(V)PGAGIPGAAVPGVVSPEAAA(K) 0	355-374
78	1696.9	1696.8945	(K)LPGGYGLPYTTGKLPY(G)	213-228
79	1702.2	1702.001	(G)VPGVGGLGGIPPAAAAKAAK(Y)	719-738
80	1706.8	1706.8424	(P)GGYGLPYTTGKLPYGY(G)	215-230
81	1718.9	1718.9072	(G)GVPGVGGVPGVGISPEAQAA(A)	425-444
82	1758.9	1758.8882	(G)VGAGVPGFGAGADEGVRRS(L)	602-620
83	1762.9	1762.985	(K)AAQFGLVPGVGVAPGVGVAP(G)	497-516
84	1763.9	1763.8639	(P)GGYGLPYTTGKLPYGYG(P)	215-231
85	1770.0	1770.0021	(A)AKAAKYGARPGVGVGGIPT(Y)	378-396
86	1832.9	1833.0229	I)PGAAVPGVVSPEAAAKAAAKA(A)	360-380
87	1840.0	1840.0000	(A)AAAAAKAAKYGAAAGLVPGGPG(F)	307-328
88	1851.0	1850.9912	(S)RPGGVPGAIPGGVPGGVFYP(G)	25-44
89	1885.0	1884.9967	(Y)GVGAGGFPGFGVGVGGIPGVAGV(P)	398-420
90	1920.0	1920.0338	(V)PGLGVGAGVPGLGVGAGVPGFGAG(A)	589-612
91	1929.8	1930.0181	(P)GGVFYPGAGLGALGGGALGPGGK(P)	39-61
92	1942.0	1942.0294	L)GGVLGGAGQFPLGGVAARPGFG(L)	745-766
93	1998.1	1998.092	(G)LGGVLGGAGQFPLGGVAARPGF(G)	746-765
94	2168.1	2168.069	(F)GAGADEGVRRSLSPELREGDP(S)	610-630
95	2367.2	2367.188	(K)YGARPGVGVGGIPTYGVGAGGFPGFG(V)	383-408
96	2620.3	2620.3074	(D)EGVRRSLSPELREGDPSSSQHLPS(T)	615-638
97	2823.5	2823.5304	A)GLGGVLGGAGQFPLGGVAARPGFGLSPIFPG(G)	743-773

Appendix B

Neutrophil elastase amino acid sequence from UniProtKB database, P08246

```
>sp|P08246|ELNE_HUMAN Neutrophil elastase OS=Homo sapiens GN=ELANE PE=1 SV=1
MTLGRRLACLFLACVLPALLLGGTALASEIVGGRRARPHAWPFMVSLQLRGGHFCGATLI
APNFVMSAAHCVANVNVRAVRVVLGAHNLSRREPTRQVFAVQRIFENGYDPVNLLNDIVI
LQLNGSATINANVQVAQLPAQGRRLGNGVQCLAMGWGLLGRNRGIASVLQELNVTVVTSL
CRRSNVCTLVRGRQAGVCFGDSGSPLVCNGLIHGIASFVRGGCASGLYPDAFAPVAQFVN
WIDSIIQRSEDNPCPHPRDPDPASRTH
```

MMP12 sequence from UniProtKB database, P39900

```
>sp|P39900|MMP12_HUMAN Macrophage metalloelastase OS=Homo sapiens GN=MMP12
PE=1 SV=1
MKFLLILLLQATASGALPLNSSTSLEKNNVLFGERYLEKFYGLEINKLPVTKMKYSGNLM
KEKIQEMQHFLGLKVTGQLDTSTLEMMHAPRCGVPDVHHFREMPGGPVWRKHYITYRINN
YTPDMNREDVDYAIRKAFQVWSNVTPLKFSKINTGMADILVVFARGAHGDFHAFDGKGGI
LAHAFGPGSGIGGDAHFDEDEFWTTHSGGTNLFLTAVHEIGHSLGLGHSSDPKAVMFPTY
KYVDINTFRLSADDIRGIQSLYGDPKENQRLPNPDNSEPALCDPNLSFDAVTTVGNKIFF
FKDRFFWLKVSERPKTSVNLISSLWPTLPSGIEAAYEIEARNQVFLFKDDKYWLISNLRP
EPNYPKSIHSFGFPNFVKKIDAAVFNPRFYRTYFFVDNQYWRYDERRQMMDPGYPKLITK
NFQGIGPKIDAVFYSKNKYYYFFQGSNQFEYDFLLQRITKTLKSNSWFGC
```

Alignment of neutrophil elastase and MMP12 amino acid sequences

```
CLUSTAL 2.0.12 multiple sequence alignment


Neutrophil elastase             MTLGRRLACLFLACVLPALLLGG--TALASEIVGGRRARPHAWPFMVS-L 47
MMP12                           --MKFLLILLLQATASGALPLNSSTSLEKNNVLFGERYLEKFYGLEINKL 48
                                  :   *  *: * .  ** *..  :   .::: *.*   : : : :. *

Neutrophil elastase             QLRGGHFCG-------------------ATLIAPNFVMSAAHCVANVNVR 78
MMP12                           PVTKMKYSGNLMKEKIQEMQHFLGLKVTGQLDTSTLEMMHAPRCGVPDVH 98
                                 :   ::.*                   . * :..: *  *   .  :*:

Neutrophil elastase             AVRVVLG----------------AHNLSRREPTRQVFAVQRIFENG---- 108
MMP12                           HFREMPGGPVWRKHYITYRINNYTPDMNREDVDYAIRKAFQVWSNVTPLK 148
                                 .* : *                : ::.*.:    :  . :::.*

Neutrophil elastase             YDPVN--LLNDIVILQLNGSATINAN----VQVAQLPAQGRRLG------ 146
MMP12                           FSKINTGMADILVVFARGAHGDFHAFDGKGGILAHAFGPGSGIGGDAHFD 198
                                :. :*  : : :*::  .. . ::*       :*:  . *  :*

Neutrophil elastase             ---------NGVQCLAMGWGLLGRNRGIASVLQELNVTVVTSLCRRSNVC 187
MMP12                           EDEFWTTHSGGTNLFLTAVHEIGHSLGLGHSSDPKAVMFPTYKYVDINTF 248
                                         .*.: :  .   :*:. *:.   :  * . *      *.

Neutrophil elastase             TLVRGRQAGVC--FGD-----------SGSPLVCN--------------- 209
MMP12                           RLSADDIRGIQSLYGDPKENQRLPNPDNSEPALCDPNLSFDAVTTVGNKI 298
                                 *  .   *:   :**           ...* :*:

Neutrophil elastase             -------------------GLIHGIASFVRGGCASGLYPDAFAPVAQFV 239
MMP12                           FFFKDRFFWLKVSERPKTSVNLISSLWPTLPSGIEAAYEIEARNQVFLFK 348
                                     .** .: . : .*  :.   :*   *  *
```

Appendix B (cont.)

```
Neutrophil elastase             N---WIDSIIQRSEDNP---------------------------------- 253
MMP12                           DDKYWLISNLRPEPNYPKSIHSFGFPNFVKKIDAAVFNPRFYRTYFFVDN 398
                                :   *: * :: . : *

Neutrophil elastase             ----------CPHPRDPDPASRTH-------------------------- 267
MMP12                           QYWRYDERRQMMDPGYPKLITKNFQGIGPKIDAVFYSKNKYYYFFQGSNQ 448
                                           .*  *.  ::..

Neutrophil elastase             ----------------------
MMP12                           FEYDFLLQRITKTLKSNSWFGC 470
```

MALDI-TOF

Proteins and peptides have been characterized by high pressure liquid chromatography (HPLC) or SDS PAGE by generating peptide maps. These peptide maps have been used as fingerprints of protein or as a tool to know the purity of a known protein in a known sample. Mass spectrometry gives a peptide map when proteins are digested with amino end specific, carboxy end specific, or amino acid specific digestive enzymes. This peptide map can be used to search a sequence database to find a good match from the existing database. This is because the more accurately the peptide masses are known, the less chance there is of bad matches.

Introduction

Electron spray ionization coupled to triple quadrupole (TSQ) and ion trap mass spectrometers (ITMS) and matrix assisted laser desorption ionization (MALDI) coupled to time of flight (TOF) analyzers have been successful for obtaining very accurate mass measurements. TOF, TSQ, and ITMS can give mass accuracies better than 0.1. MALDI-TOF mass spectra (MS) is a good tool for screening peptide masses of tryptic digests. This method is more effective because it requires relatively less intense sample preparation since the matrix is less susceptible to interferences caused by salts and detergents. Secondly MALDI-TOF-MS generates peptides containing only one charge and show only one peak in spectrum which facilitates data interpretation.

Schematic and Theory of MALDI

MALDI is a very sensitive technique for determining the mass of proteins, peptides, or polymers. Protein masses are identity of proteins and thus help in proteomics. Thus MALDI allows protein identification. MALDI sample preparation is relatively fast and easy. It is a first choice when it comes to protein study. Proteins, peptides, and polymers are fragile and tend to fragment when ionized by other ionization techniques.

1,2.bmp

MALDI is attached to a time of flight (TOF) analyzer which measures time it takes for the molecules to travel a fixed distance. MALDI is a soft ionization technique in which a short laser pulse, instead of continuous laser, of nitrogen gas usually around 237 nm is used to ionize molecules. A protein or peptide sample is placed on a target plate and mixed with an appropriate matrix on the target plate. The mixture of sample and matrix crystallizes due to the vacuum environment and then is irradiated with a short laser pulse. The sample molecules and the matrix now enter gas phase. This leads to release of matrix, samples molecules, and ions from the target plate. The ions then accelerate in TOF analyzer because they are subject to equal electric field. TOF is a field-free flight tube. The ions travel in a strait and linear direction to the detector. The mass to charge ration (m/z) of the sample ions can be calculated using the equation $T= C1(m/z)^{0.5} +C2$. C1 and C2 are instrumental constants which can be determined with compounds of known mass. This equation is derived from the fact that potential energy equals kinetic energy.

$$KE = 0.5mv^2 \qquad \text{eq. 1}$$

$$v = (2KE/m)^{1/2} \qquad \text{eq. 2}$$

Since velocity is distance/time substitute it in eq. 2 and solve for t to get eq. 3

$$t = m^{1/2} * d/(2KE)^{1/2} \qquad \text{eq.3}$$

The distance the molecules travel and their kenetic energy is constant. So it is replaced by C1. Futhermore since the relationship between the t and $m^{1/2}$ is linear an intercept of C2 is added to get eqation of a line.

$$t = C1(m/z)^{1/2} + C2 \qquad \text{eq.4}$$

Sample preparation

Biomolecules such as proteins, peptides, sugars, and large organic molecules such as polymers, dendrimers and other macro molecules can be analyzed using MALDI. Sample preparation for MALDI is very simple; however, it is one of the most crucial steps in the MALDI analysis process. MALDI is more tolerant to sample contaminants, but contaminants can seriously disturb incorporation of sample molecules with growing matrix crystals. This results in bad spots on the target plate, leading to low signal to noise ratio, resolution, and sensitivity.

Samples can be prepared in two different ways. One removes the contaminants before applying them on to the matrix and one removes the contaminants after the sample is spotted on to the target plate either before or after adding the matrix.

Miniaturized chromatographic set-up is used for the first approach, while some scientists have cleaned in-gel digest of proteins using reverse phase(RP) HPLC microcolumns packed with different types of RP-HPLC beads (1). Now tips

are also packed with RP or ion-exchange resin to remove salts and detergents from protein mixtures, and their effectiveness is shown by their recent commercialization. Tips and columns effectively remove MALDI contaminants and give small volume of sample, this can result in possible highly sensitive MALDI analysis of the samples.

Purification of biological samples on target plates involves synthetic membranes or surfaces. Membranes go on top of the target plate and the biological samples are spotted on top of the membranes. Biological samples interact with the membrane through strong hydrophobic forces. This enables samples to remain on the membrane while the buffers and salts are washed away. Then the MALDI-matrix solution is added to the purified samples on the target plate, ready for analysis. Perfluorosulfonated ionomer films, polyethylene membranes, nonporous polyurethane membranes, and C8 and C18 extraction disks are examples of membranes that have been used successfully in the past for biological mixtures. Self-assembled monolayers (SAMs) of octadecyl mercaptan on gold-sputtered disposable MALDI probe tips have been used to concentrate the sample and to act as a purification device. However, it is unfeasible because it requires overnight sample incubation to fully concentrate at the probe tip.

Matrix

The function of the matrix is adsorborption of energy from laser pulse, and then transfer to sample"thereby causing desorption of the analyte molecules in an expanding plume, to ionize the desorbed analyte molecules and to prevent aggregation of the analyte molecules"(2). The matrix molecules for MALDI are chosen on the basis on fulfillment of requirement that matrix molecules must be able to absorb ultra violet wavelength of usually 237nm, low volatility and ability to transfer protons to the sample molecules. For proteins samples typical MALDI matrix consist of cinnamic acid and hydroxylated benzoic acid derivatives.

2,5-dihydroxybenzoic acid is more tolerant to the sample contaminants because it excludes them during crystallization process(3). The use of specially prepared thin matrix layers uses fast evaporation setup, which not only improves the sensitivity and resolution, but also allows the samples to be extensively washed, removing salts and detergents. Since the sensitivity depends on the concentration of the sample on the target plate, samples can be concentrated using PR-HPLC or bead-peptide concentration. In bead-peptide concentration RP-chromatographic beads are added to the proteins or peptide samples, and these samples preferentially bind to the beads through hydrophobic interactions while the contaminants like salts and chaotrophes do not. After a short incubation, the peptide-bead solution is harvested using pellets from a centrifugation, and dried in speed vacuum concentrator. In both cases, highly concentrated pellets of peptide-bound beads are obtained, which can be transferred to MALDI and left to dry. Because the beads are hydrophobic in nature, they form a cluster in highly concentrated spot (<1mm^2) on MALDI target plate after drying. Peptides elute on the target plate by a small volume of aqueous/organic MALDI-matrix solution and become incorporated into the growing matrix crystals at the same time. This process allows 10 to 100 fmol to be enough to be loaded on to the gels.

Sensitivity

Sensitivity of MALDI depends on sample preparation and the preparation of sample/matrix layer. They must be optimized by trial and error according to sample size, type, and previous history. Preparing a very thin matrix layer and applying of sample so that the sample is on the outer layer of matrix, give a sensitivity level of low attomole (10^-18). This method makes it possible for "...removal of salts present in the analyte solution by a sample washing procedure away from the sample very simply" (4). Small matrix spots using nanoliter volumes of matrix and analyte solution, combined purification, concentration, and application procedures have also given similar sensitivities (5). In this method, matrix is adsorbed to nanoliter bed volume reversed-phase column prepared in an Eppendorf GeLoder tip. Then the column is washed and the sample is eluted with a few nanoliter volume of matrix onto the target plate.

Sensitivity is reduced with increasing molecular weight. The sensitivity is two to three magnitudes lower for proteins than it is for peptides. So the sensitivity of proteins is in the femtomole range.

Structural Information

Structural information of proteins can be determined by digesting proteins with specific endoprotease like trypsin, AspN, and GluC. MALDI is one of the best spectrometric techniques for direct analysis of peptide mixtures. Signals of peptides are suppressed because there is a competition for charge or optimal position in the matrix. Therefore signal intensity does not necessarily reflect the quantities of different peptides in the mixtures. Complete sequences can be obtained from a combination of spectra recorded in different modes, like positive and negative, matrices, and different enzyme digestion.

Sequence information is also possible to get from PSD. This is possible by controlling the voltage of the reflector, which results in different m/z ranges on the detector and generates a PSD spectrum. A large sample amount is required since only a small fraction goes under PSD. Additionally, the fragmentation can not be controlled since different site of a peptide can get fragmented. This makes it very had to get complete sequences of a peptide. Alternatively, collision cells are included to the flight tube in MALDI-TOF by some manufacturers, to have controlled fragmentation by collision-induced dissociation.

Large amounts of in-source fragmentation occurs before initiation of the acceleration voltage called in-source decay in delayed extraction equipped MALDI-TOF, which only yield long regions of sequence-specific ions (6). C-terminal sequence ladders can be generated by digestion of peptides with carboxypepsidase and N-terminal sequence ladders can be obtained by Edman degradation using low percentage of phenyltiocarbamate rather than phenyoisothiocarbamate in the coupling reaction. These ladders in the mixtures of peptides can be an alternative to sequence-specific fragment ions. This process often gives a lot of sequence information.

Secondary protein modifications can also be determined using MALDI-TOF-MS. The steps involved in determining secondary modifications are measuring mass of the intact protein, knowing the protein's primary sequence, and generating site-specific information by direct mass spectrometric peptide mapping of a mixture derived by proteolytic cleavage of the proteins. In tandem (TOF/TOF) configurations, MALDI instruments can provide protein sequence data, as well.

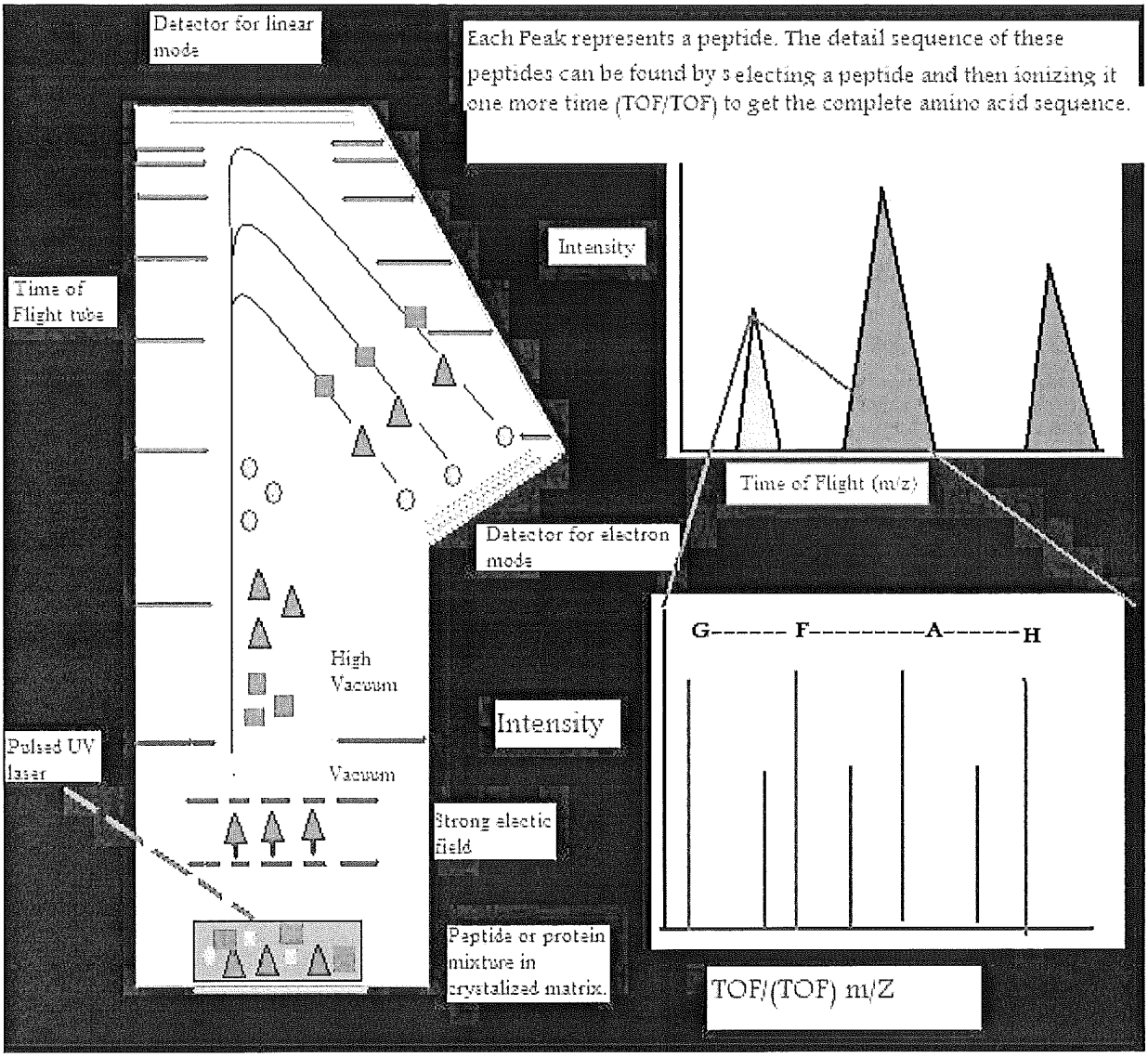
In tandem mass spectrometry, an ion of a particular mass is selected (that's the first stage of the analysis) and fragmented. Its constituent fragment ions are then mass-analyzed a second time (that's the tandem stage) to reveal data about the molecule's structure or sequence; single-stage TOF instruments lack this capability (though some fragmentation does occur via "post-source decay" as the ions traverse the flight tube).

Some companies offer tandem MALDI instruments based on hybrid mass analyzer configurations. Applied Biosystems' QSTAR, for instance, couples an optional MALDI source with a quadrupole-time-of-flight mass analyzer, as does Waters Corporation's MALDI Q-Tof Premier.

TOF analyzer

Modes

Ions can travel in a linear fashion and be detected by the detector at the opposite end as an ion source. This is called linear TOF. It is different from a reflectron TOF, in which ions are reflected to electrostatic mirror and detected by another detector. Linear TOF spectrum is limited in resolution leading to low mass accuracy. This is because initially different amount of kinetic energy can be attained by the ions with the same charge. This leads to different (m/z) ratio of ions which have different initial velocities. This is partially corrected by reflectron TOF. High energy ions penetrate deeper into the reflectron, taking longer distance and time, while low energy ions do not penetrate as deep into the reflectron and take a shorter path and time. This leads to correction of different times of ions with the same mass and charge. This leads to an increase of resolution to 10,000.



The amount of energy put into ions by the laser initially can also be corrected by a technique called delayed extraction (DE) in which acceleration voltage is applied slightly after the laser pulse. DE TOF increases resolution to 2000.

RE TOF can give full isotopic resolution for molecules up to 15 kDa. However 12C-only ion peak will have very low intensity for molecules bigger than 5kDa. Resolution is further decreased because each ion decays after acceleration and its time of flight can not be adjusted by RE TOF because decay has already occurred. These ions are not detected in RE TOF. However, these ions can be detected in linear TOF, but resolution still decreases because linear TOF has no way of correcting different energy inputs to an ion with the same mass and charge. It is better to use linear mode to get spectra and determine isotopically averaged mass for molecules bigger than 5-10 kDa. Resolution depends on the size of the molecules in a sample. The greater the size that the sample molecule has, the lower the resolution of it.

MALDI-TOF can only be compared to ESI because they are two ways of directly analyzing proteins, peptides, and polymers. MALDI-TOF samples can be reanalyzed while ESI samples can not because ESI is connected to LC column, and the analysis is limited to the width of the chromatographic peak. MALDI-TOF can scan 10 spectras for a peak 10 seconds wide per second, while it takes ESI almost 15 second. MALDI-TOF-MS generates mostly ions +/- 1 charge while ESI generates a charge for every 8-10 amino acids. That means ESI spectra are considerably more complex than MALDI spectra. Coulombic repulsion increases as the charge increase, leading to data deviation, and in MALDI

spectra, this repulsion does not occur. However, multiple charges in ESI give better resolution because the higher the mass to charge ratio, the harder it is to get good resolution. In general MALDI is faster than ESI, and enables higher throughput. But ESI is more sensitive.

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